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The effect of light and heterotrophy on carotenoid concentrations in the Caribbean anemone *Aiptasia pallida* (Verrill)

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Abstract One of the consequences of ultraviolet radiation exposure in anthozoans possessing photosynthetic endosymbionts (i.e., zooxanthellae) is production of reactive oxygen species that can damage cellular components, especially lipids and photosynthetic membranes. It is well known that carotenoids are potent antioxidants that can mitigate oxygen radical damage, but the origin of these compounds in zooxanthellate anthozoans is obscured because they can potentially originate from endosymbionts, heterotrophic feeding by the host, or a combination thereof. We used *Aiptasia pallida*, a common Caribbean anemone, to investigate the effects of exogenous food sources, ultraviolet-A radiation (UVA, 320–400 nm), and photosynthetically active radiation (PAR, 400–700 nm) on carotenoid levels in zooxanthellate anthozoans. Anemones were exposed to one of three simulated light treatments in the laboratory for 38 days: PAR (60% below ambient)/UVA (similar to ambient), PAR/low UVA (42% below ambient), and low PAR (98.4% below ambient)/no UVA. In addition, anemones were either fed a carotenoid-rich diet of *Artemia salina* nauplii, or starved. Carotenoids identified in *A. pallida* included peridinin, diadinoxanthin, diatoxanthin and β-carotene. While a diet of *Ar. salina* nauplii had no effect on the carotenoid composition of *A. pallida*, a two-way analysis of variance revealed that anemones exposed to ambient UVA levels had significantly greater diatoxanthin concentrations relative to the total xanthophyll pool [diato: (diato + diadino)] after 10 days of exposure. This difference among treatments was not present at 20 days, but reappeared as an effect due to starvation rather than UVA at days 30 and 38. These results suggest that carotenoids in *A. pallida* are not influenced by exogenous feeding and that photoprotective xanthophyll cycling is sensitive to stresses such as UVA and starvation.

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Introduction

Anthozoans, such as corals and anemones, that maintain photosynthetic, endosymbiotic dinoflagellates of the genus *Symbiodinium* (i.e., zooxanthellae) are dominant members of many tropical marine benthic ecosystems (Odum and Odum 1954). The symbiosis is a mutualistic association where algae provide the host with essential nutrients, such as amino acids and fixed organic carbon (Harland et al. 1991; Davies 1997), and the host provides zooxanthellae with nutrients, such as nitrogen and phosphorus (Davies 1997). Reliance upon products derived from photosynthesis requires that symbiotic anthozoans inhabit environments where visible light intensity promotes net primary production of the algae (Falkowski et al. 1990). Ironically, occupying these photic environments also exposes these organisms to ultraviolet (280–400 nm) radiation (Jokiel and York 1982; Shick et al. 1996).

Ultraviolet radiation (UVR) reaching the earth is particularly intense near the equator due to the thinness of the ozone layer and the low zenith angle of the sun (Baker et al. 1980). UVR can penetrate clear, nutrient-poor tropical waters to depths greater than 20 m (Fleischmann 1989; Gleason and Wellington 1993). Exposure to UVR can induce formation of oxygen radicals such as singlet oxygen ($^1O_2$), superoxide (O_2^-) and hydrogen peroxide (H_2O_2) that disrupt protein synthesis and damage cell membranes (Lesser 1996; Shick et al. 1996). Moreover, these radicals may damage photosynthetic membranes resulting in decreased

photosynthetic rates (Lesser and Shick 1989; Lesser et al. 1990; Lesser 1996, 1997).

Symbiotic organisms may ameliorate themselves from the deleterious effects of UVR exposure with carotenoids that quench oxygen radicals present in both algal and host animal cells. During zooxanthellar photosynthesis, excess photochemical energy can excite chlorophyll molecules into a triplet state that promotes the formation of damaging singlet state oxygen radicals when the energy is transferred to ground state oxygen (Porra et al. 1997). The xanthophyll cycle carotenoid, diadinoxanthin, accepts excess chlorophyll excitation energy and forms its sister compound diatoxanthin through a light-dependent de-epoxidation reaction. This enzymatic reaction mediated by violaxanthin de-epoxidase, readily dissipates excess light energy as heat, thereby providing a rapid, recyclable mechanism to protect photosynthetic membranes against photoinhibition that may damage or destroy algal cells (Will et al. 1989; Frank et al. 1996; Porra et al. 1997; Brown et al. 1999). Carotenoids, such as β-carotene, that may be present in symbiotic plant and animal cells may also act to quench singlet oxygen based on the highly congegated double bond system of the carotenoid molecule (Will and Scovel 1989; Porra et al. 1997).

Only plants and some bacteria and fungi can synthesize carotenoids de novo; hence animals can only obtain carotenoids through their diet (Goodwin 1980). Carotenoids are fat-soluble (Olsen and Owens 1998) and may be transferred to animals through dietary intake of associated lipids and proteins (Schiedt 1989). In symbiotic anthozoans, up to 30% of the organic carbon from photosynthesis may be translocated directly from the zooxanthellae to the animal host in the form of lipids (Patton and Burris 1983). Thus, carotenoids may be extruded or leaked from zooxanthellae into intracellular spaces or may be sequestered by host cells. Moreover, many symbiotic anthozoans supplement the dietary contributions of zooxanthellae by consuming crustaceans and other zooplankton known to contain carotenoids (Kleppel et al. 1988; Sebens et al. 1996). As a result, the animal host may obtain carotenoids from both zooxanthellae and by feeding on carotenoid-rich zooplankton. These multiple modes of nutrition obscure determination of the origin of these compounds in tissues of symbiotic anthozoans.

Recent studies of reef-building corals have made considerable progress in understanding the role of carotenoids in protecting the photosynthetic apparatus of the zooxanthellae from irreversible light-induced photoinhibition that may lead to the loss or expulsion of the algal symbiont from the cnidarian host (Ambarsari et al. 1997; Brown et al. 1999). However, these studies have not considered the possibility that carotenoids might be derived from sources other than the zooxanthellae, such as heterotrophic sources, and that carotenoids might also be important for removing reactive oxygen species produced from non-photosynthetic hyperoxia in animal tissues. Indeed free-radicals and other active oxygen species are natural by-products of biological redox reactions and can be present in all tissues where antioxidant enzymes, such as superoxide dismutase, carotenoids and other antioxidation reactions may moderate their detrimental effects (Cockell and Knowland 1999).

The primary goal of this study was to investigate the contribution of heterotrophic feeding to carotenoid pigmentation in the tissues of the common symbiotic Caribbean sea anemone, *Aiptasia pallida* (Verrill). Secondarily, we partitioned the effects of photosynthetically active radiation (PAR: 400–700 nm) and UVA radiation on the concentration of carotenoids in zooxanthellae and the cnidarian host.

Materials and methods

Collection and acclimatization of anemones

Aiptasia pallida (Verrill) occurs in shallow water throughout Florida and the Caribbean (Kaplan 1982). Specimens of *A. pallida* used in this study were collected from a flow-through seawater system shaded from direct solar irradiance at the Keys Marine Laboratory, Long Key, Fla. (24°49.55′′N, 80°48.88′′W) and waters under a dock adjacent to the Key Largo Marine Research Station, Key Largo, Fla. (25°06′04′′N, 80°26′19′′W) during 24–25 November 1999 and 13–17 March 2000.

Anemones were transported to Georgia Southern University in 20 l buckets containing ambient sea water with aerators. Anemones were transferred into two 46 l glass aquariums containing UVC (< 280 nm) sterilized artificial seawater (Instant Ocean; 35‰) housed in a climate-controlled room (25°C). Two weeks prior to the experiment, each anemone was transferred into a clean, white 70 ml polystyrene medical cup (Solo) filled with 50 ml of sterilized artificial seawater. Anemones were allowed to acclimatize during this period to the experimental PAR (400–700 nm) photoperiod of 12 h light: 12 h dark. Light was supplied by a bank of 12 20-W daylight fluorescent lights (Sylvania Co., Sylvania, Ga.) at an instantaneous irradiance of 20 μmol m^{-2} s^{-1} as measured by a LI-Cor LI-190SA quantum sensor and LI-Cor LI-1000 data logger (LI-Cor, Inc., Lincoln, Neb.). During the acclimatization period, anemones were fed to repletion every other day with live *Ar. salina* (San Francisco Brand Brine Shrimp) nauplii raised in artificial seawater. Water was changed the day after feeding to remove debris.

Experimental design

Each of six independent light units consisted of a combination of two 20-watt daylight (total combined irradiance of 20 μmol m^{-2} sec^{-1}; light emission range = 400–700 nm) and two 20-watt UVA fluorescent lamps (λ_{max} = 360 nm, light emission range = 310–400 nm; Ultraviolet Resources International, Cleveland, OH). Light units were separated with thick black vinyl to prevent leakage of light to adjacent experimental units. Groups consisting of four anemones representing four sampling times (10, 20, 30 and 38 days) were randomly assigned to one of five treatments (n = 6 per treatment) under each light unit: (1) low PAR [98.4% below ambient]/no UVA/fed; (2) PAR [60% below ambient]/low UVA [42% below ambient]/fed; (3) PAR/low UVA/unfed; (4) PAR/UVA [similar to ambient]/fed; and (5) PAR/UVA/unfed. Unfed treatments were intentionally omitted in low PAR treatments because preliminary experiments under these conditions resulted in atrophied anemones and, in some instances, death.

Treatments were interspersed by lottery under each light unit. Anemones in PAR/UVA treatments were covered with a 14×14×0.3 cm piece of UVR transmitting acrylic (mean %

transmission of UVB ~12, UVA ~33, PAR ~90). Anemones in PAR/UVA treatments received UVA intensities similar to those encountered in the field, while PAR intensities were 60% below ambient as measured with a LI-Cor LI-1800UW scanning spectroradiometer (Table 1). PAR/low UVA treatments were covered with UVR blocking acrylic (mean % transmission of UVB ~0.3, UVA ~23, PAR ~87) that reduced incoming UVA radiation by 42% (Table 1). Anemones in the PAR/low UVA treatments were exposed to a 60% reduction in PAR and 42% reduction in UVA compared to intensities encountered in the field (Table 1). Low PAR/no UVA treatments were covered with a piece of plexiglas wrapped in aluminum foil. This treatment reduced PAR by 95% compared to the PAR treatment and was 99.9% lower than those intensities encountered in the field (Table 1).

The experiment ran for 38 days and anemones in fed treatments received equal portions of *Ar. salina* nauplii ad libitum every other day. Although this does not represent a natural diet, distributing food equally in all fed treatments controlled for carotenoid supplementation. Water was replaced with oxygenated, UVC-sterilized, artificial seawater on days following host feeding for all anemones. Plastic containers were scrubbed with a soft bristle brush every seven days to remove algae and debris.

Anemones were sampled between 1200 and 1400 h, corresponding to the daily median light exposure and when zooxanthellae division rates are lowest for laboratory raised *A. pallida* and other symbiotic species (Fitt 2000). Anemones were starved for 24 h prior to sampling to rid them of digested food in vacuoles (Clayton and Lasker 1984; Fitt 2000). Twelve anemones were chosen randomly prior to initiation of the experiment and analyzed for carotenoid concentrations per zooxanthella and per mg protein. Subsequently, one anemone from each treatment was selected randomly for analysis on days 10, 20, 30 and 38. Anemones were processed for pigment concentration and zooxanthellae density in dim light immediately following collection. Whole anemones were homogenized with a Teflon pestle in a 1.5 ml microcentrifuge tube for one minute and tissue sub-samples were removed for zooxanthellae density and extracted for pigment content analysis.

Homogenized tissue to be used for quantification of zooxanthella densities was placed in 1.5 ml microcentrifuge tubes containing 0.5 ml of 95% filtered artificial seawater and 5% formalin. A 0.2-ml aliquot was removed for protein analysis and the remaining tissue was diluted to an appropriate volume for accurate zooxanthellae counts using a hemacytometer and a compound microscope. Six replicate counts were recorded for each sample and the means were standardized to mg of soluble protein assayed using the Bradford technique (1976).

Pigments were extracted by transferring anemone tissue into a 1.5 ml microcentrifuge tube containing 100%, degassed, HPLC grade methanol and placing these tubes immediately into a −80°C freezer. Anemone tissue was extracted once for 24 h and was centrifuged at 5,000 RPMs for 20 min at 4°C. The resulting supernatant was transferred to an amber Waters autosampler vial and stored at −80°C prior to HPLC analysis. The remaining tissue pellet was stored at −20°C for protein analysis.

Carotenoids were extracted from *Ar. salina* nauplii and cysts by filtering cultures raised in artificial seawater with a 70 μm sieve followed by washing with 100 ml of ddH_20. Nauplii and cysts were then transferred to a 1.5 ml microcentrifuge tube and ground with a Teflon pestle in 1 ml of 100% HPLC grade acetone. Since the majority of carotenoids in *Ar. salina* nauplii and cysts are bound to lipophyllic integumentary structures, acetone was used to extract carotenoids rather than methanol. The homogenized nauplii and cysts were then extracted for 24 h at −80°C, centrifuged at 15,000 RPMs for 5 min at 4°C and transferred to an amber Waters autosampler vial and stored at −80°C prior to HPLC analysis. Carotenoids from nauplii and cysts were co-chromatographed with anemone tissue to identify any shared carotenoid species.

Pigments were analyzed using reverse-phase HPLC on either a Waters C_{18} Lichrosorb 5 μm (25 cm×0.46 cm) column, protected by a Waters Lichrosorb guard column or a Phenomenex C_{18} Lichrosorb 5 μm (25 cm×0.46 cm) column, protected by a Phenomenex Security Guard Cartridge System. A binary gradient solvent system was employed consisting of solvent A (80% methanol and 20% 1 N ammonium acetate) and solvent B (60% methanol and 40% acetone). Pigments were separated by a linear gradient from 100% A, 0% B to 100% B, 0% A over 10 min followed by an isocratic hold at 100% B for 10.0 min (12.5 min for Phenomenex column) at a flow rate of 1 ml min^{-1} according to Ambasari et al. (1997). Pigments were monitored at 440 nm using a Waters model 490E multiple wavelength UV/VIS detector.

Identification of pigments was accomplished by spectrophotometric peak confirmation with known standards. Quantification of pigment content was based on peak area calibration to chromatographed standards. Peridinin, diadinoxanthin, and diatoxanthin standards of known concentrations (mg/l) were obtained from the International Agency for ^{14}C Determination, Denmark. Concentrations of β-carotene (all trans β, β-carotene) dissolved in 100% acetone were determined by spectrophotometery using published molar extinction coefficients at wavelengths of maximum absorbance from standards obtained from Sigma-Aldrich Co., USA (Jeffery & Wright 1997).

Protein assays were conducted using the Bradford technique (Bradford 1976). Protein from centrifuged tissue pellets of zooxanthellae density and pigment extractions was dissolved in 0.6 ml of 0.12 N NaOH for 60 min in a 90°C water bath. Soluble protein was quantified by spectrophotometery (595 nm) using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) and scaled to appropriate volumes using bovine gamma globulin as a standard. Pigment concentrations were standardized to mg protein initially and then compared to zooxanthella estimates [cells (mg protein)$^{-1}$] to give a pigment concentration expressed as picograms per zooxanthella.

Table 1 Radiometric data in the laboratory and at the field collection site at the Key Largo Marine Research Station. UVB, UVA and PAR intensities are based on the average of three scans taken at 2-nm intervals using a LI-Cor LI 1800UW scanning spectroradiometer and integrated over designated wavelengths. Scans in the laboratory were made 10 cm from the light source with respective filters in place 4 cm from sensor. Initial values represent scans taken without Plexiglas filters or UVA lamps. All other readings were taken under experimental conditions described in Materials and methods. The mean total integrated UVB, UVA and PAR intensities from the field are based on measurements taken on three consecutive cloudless days once every hour between 1330 and 1930 hours on 15 August, 0830 and 1930 hours on 16 August and 0830 and 1030 hours on 17 August 1999 (EST) at 1 m depth

	Intensity (W m^{-2})		
	UVB (300–320 nm)	UVA (320–400 nm)	PAR (400–700 nm)
Laboratory			
Initial	0.0	0.0	7.6
Low PAR/No UVA	0.0	0.0	0.3
PAR/Low UVA	< 0.001	0.25	7.5
PAR/UVA	< 0.001	0.46	7.4
Field			
Mean ± SE	0.0012 ± 0.0004	0.43 ± 0.07	18.5 ± 4.3

Statistical analysis

The concentrations of peridinin, diadinoxanthin, diatoxanthin and β-carotene expressed as pg per mg protein and pg per zooxanthella were compared among light and feeding treatments at 10, 20, 30 and 38 days via a two-way analysis of variance (ANOVA). The photoprotective inter-conversion of diadinoxanthin into diatoxanthin expressed as a function of the total xanthophyll pool [Diato: (Diato + Diadino)] was also compared among treatments at each sampling period via a two-way ANOVA. Wherever concentrations of pigments were not distinguishable from baseline noise on the

chromatogram, values for peak area were assigned a 0. All analyses proceeded after first transforming data by $ln(10+x)$ to satisfy assumptions of normality and equal variances. Analyses were performed with JMP IN statistical software (SAS Institute Inc., Cary, NC).

Results

Carotenoid origins in *Aiptasia pallida*

Carotenoid pigments identified in *A. pallida* included peridinin, diadinoxanthin, diatoxanthin and β-carotene and accounted for 48% ($n=190$) of all pigments detected. Chlorophyll *a* and c_2, contributed another 41.2% and together with carotenoids, accounted for 89.2% of all pigments present (Fig. 1). Other minor peaks present in *A. pallida*, although not identified, likely consisted of dinoxanthin and isomers and epimers of chlorophylls and carotenoids, present as either full compounds or artifacts of extraction and storage (Jeffery and Wright 1997).

Extracts of *Artemia* cysts and nauplii revealed three pigment peaks (Fig. 1). Although not identified, two of these likely correspond to the carotenoids echinenone and canthaxanthin that have previously been reported in *Ar. uromiana* nauplii and cysts (Ahmadi et al. 1990). When co-chromatographed with *A. pallida*, there was no co-elution of peaks with *Ar. salina* cysts and nauplii (Fig. 1).

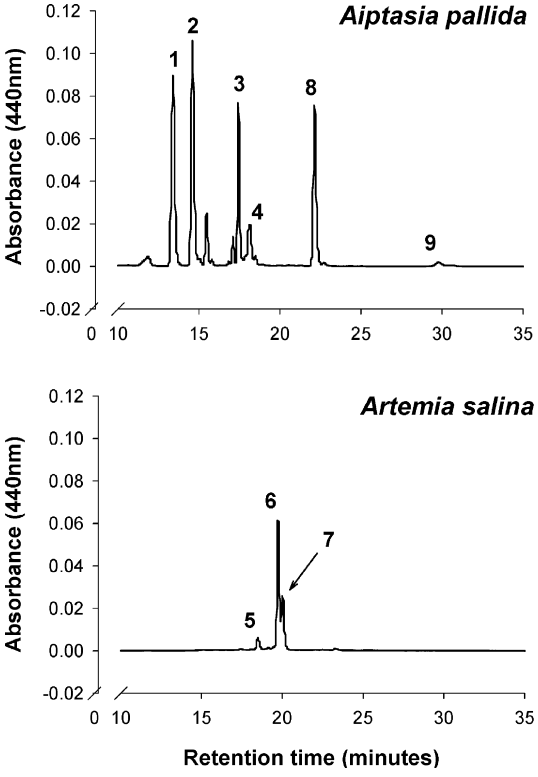


Fig. 1 Chromatogram of pigments isolated from *Aiptasia pallida* and its heterotrophic food source, *Artemia salina* nauplii. Peak number, pigment, retention time (min): 1, chlorophyll c_2, 13.467; 2, peridinin, 14.633; 3, diadinoxanthin, 17.450; 4, diatoxanthin, 18.117; 5, unknown, 18.567; 6, unknown, 19.733; 7, unknown, 20.017; 8, chlorophyll *a*, 22.017; 9, β-carotene, 29.883

Effect of light and feeding on carotenoids

UVA comprised, on average, 2% of total solar irradiance reaching anemones in field conditions, while UVB intensities were negligible (Table 1). Although experimental UVA intensities were close to mean in situ levels, PAR reaching experimental anemones was only a third of that experienced by anemones under field conditions. Given that little UVB was found in the field and preliminary experiments showed that the addition of UVB reduced anemone survival, these wavelengths were not included in the experiment.

Peridinin is the characteristic carotenoid pigment of dinoflagellates and concentrations of this compound per milligram of protein and per zooxanthella tended to be greatest in the low PAR/no UVA/fed treatment throughout the experiment (Fig. 2). However, concentrations were highly variable and significant differences in peridinin per unit of protein were detected only on day 38 (ANOVA $F_{1,22}=4.1587$, $P=0.0294$) when most treatments showed their lowest peridinin levels (Fig. 2). Concentrations of diadinoxanthin, diatoxanthin and β-carotene per milligram of protein and per zooxanthella cell followed trends similar to those encountered in peridinin with greater concentrations of carotenoids in the low PAR/no UVA/fed treatment (Figs. 3, 4, 5). Again, concentrations were highly variable and neither diadinoxanthin nor its sister compound diatoxanthin showed significant differences among light and feeding treatments for the course of the experiment (Figs. 3, 4). Concentrations of β-carotene, on the other hand, were significantly higher per unit of protein in low PAR/no UVA/fed treatments at 10, 20 and 38 days (10 days: ANOVA $F_{1,25}=4.1154$, $P=0.0285$; 20 days: ANOVA $F_{1,25}=6.1689$, $P=0.0066$, 38 days: ANOVA $F_{1,22}=5.8409$, $P=0.0092$) and per cell at 20 days (20 days: ANOVA $F_{1,23}=6.2911$, $P=0.0066$, Fig. 5).

Although there were no significant differences in diatoxanthin or diadinoxanthin concentrations among treatments (per unit of protein and per zooxanthella), the ratio of diatoxanthin to the entire xanthophyll pool was significantly greater at day 10 in treatments where both PAR and higher intensities of UVA were present (10 days: ANOVA $F_{1,25}=4.4212$, $P=0.0227$, Fig. 6). Interestingly, this light-induced effect on xanthophyll activity was independent of feeding regime on day 10, but by day 20 was replaced by a feeding effect whereby unfed treatments had a significantly higher ratio of diatoxanthin to the total xanthophyll pool (20 days: ANOVA $F_{1,25}=6.3153$, $P=0.0188$). This trend for a higher ratio in unfed treatments was maintained through days 30 and 38, but never reached significantly higher levels than fed treatments during these times.

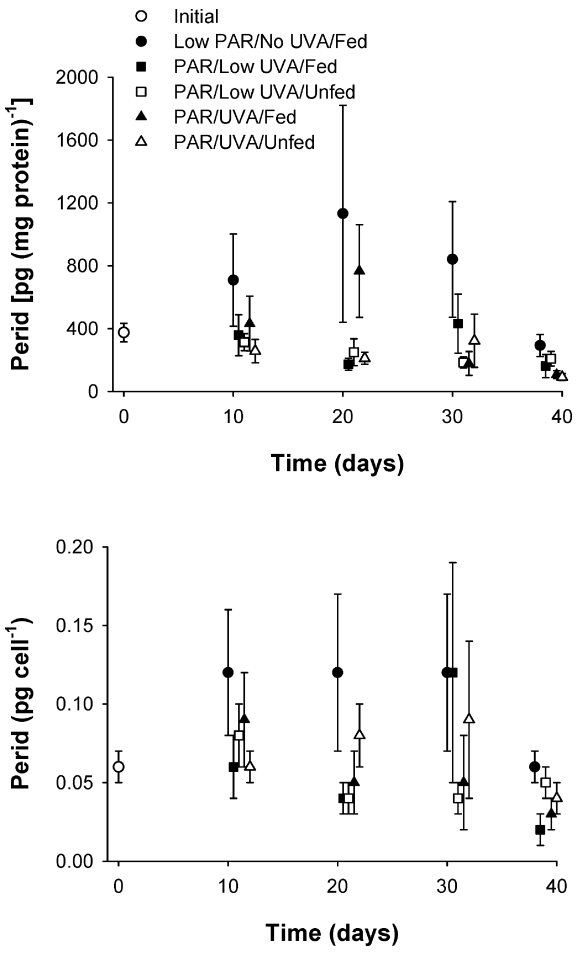


Fig. 2 Mean concentrations of peridinin per milligram of protein of anemone tissue [pg perid (mg protein)$^{-1} \pm SE$] and per zooxanthella (pg of perid cell$^{-1} \pm SE$) over 38 days in *A. pallida* receiving different levels of PAR, UVA and food. For the initial samples $n = 12$. In all treatments on subsequent days $n = 6$, except $n = 5$ as follows: low PAR/no UVA/fed for 20 and 30 days; PAR/low UVA/fed for 10, 30 and 38 days; PAR/low UVA/unfed for 20, 30 and 38 days; PAR/UVA/fed for 30 and 38 days

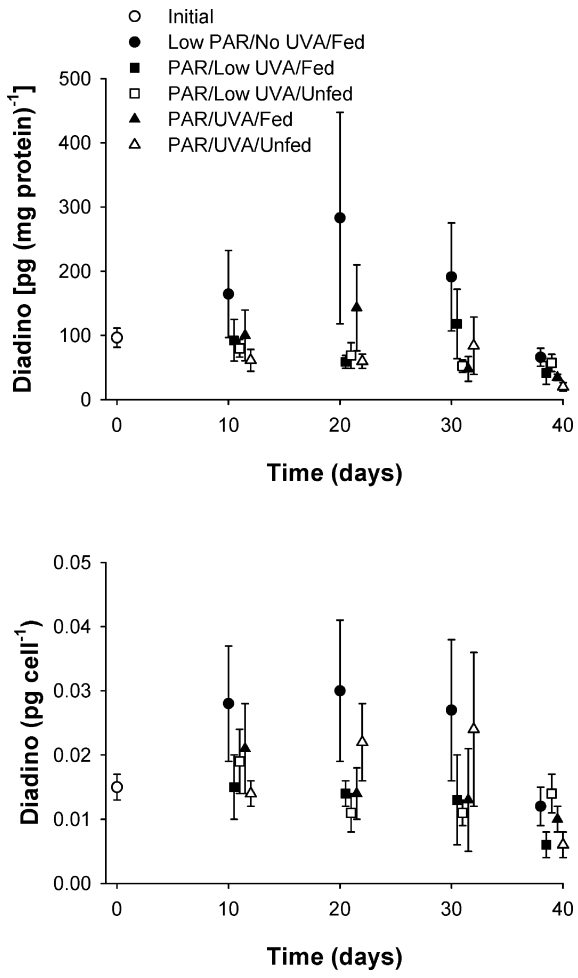


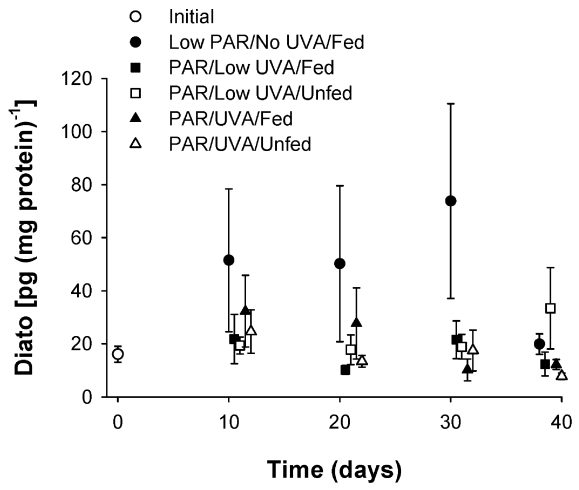
Fig. 3 Mean concentrations of diadinoxanthin per milligram of protein of anemone tissue [pg diadino (mg protein)$^{-1} \pm SE$] and per zooxanthella (pg of diadino cell$^{-1} \pm SE$) over 38 days in *A. pallida* receiving different levels of PAR, UVA and food. For [pg diadino (mg protein)$^{-1} \pm SE$], $n = 12$ for initial samples and $n = 6$ in all treatments except $n = 5$ in the following treatments: low PAR/no UVA, fed for 38 days; PAR/low UVA, fed for 10, 30 and 38 days; PAR/low UVA, unfed for 20, 30 and 38 days; PAR/UVA, fed for 30 and 38 days. For (pg of diadino cell$^{-1} \pm SE$), $n = 12$ for initial samples and $n = 6$ except $n = 5$ in the following treatments: low PAR/no UVA, fed for 38 days; PAR/low UVA, fed for 10 and 38 days; PAR/low UVA, unfed for 20, 30 and 38 days; PAR/UVA, fed for 20, 30 and 38 days; PAR/UVA, unfed for 10, 20 and 30 days; and $n = 4$ in the PAR/low UVA, fed treatment for 30 days

Discussion

Origin of carotenoids in *Aiptasia pallida*

Peridinin, dinoxanthin, diadinoxanthin, diatoxanthin and β-carotene have been isolated from the zooxanthellae of symbiotic anthozoans and mollusks (Goodwin 1980) and when combined with chlorophyll *a* and c_2, account for 95% of all pigments found in several species of symbiotic scleractinian corals (Fang et al. 1995). In this study these carotenoids comprised a similar proportion of the chromographic composition of *A. pallida* extracted from tissues (~90%), although dinoxanthin was not analyzed in this study. The pigments detected have all been associated with the light-harvesting apparatus of dinoflagellates (Jeffery and Vesk 1997; Porra et al. 1997) and have not been isolated from animal tissues lacking photosynthetic symbionts, with the exception of β-carotene (Goodwin 1984).

Pigments isolated in *Artemia salina* likely correspond to the carotenoids canthaxanthin and echinenone, which have been identified in *Ar. uromiana* nauplii and cysts (Ahmadi et al. 1990; Nelis et al. 1993). Canthaxanthin dominates the carotenoid content of *Ar. salina* cysts and nauplii while echinenone contributes a much smaller proportion (~2%) (Ahmadi et al. 1990; Nelis et al. 1993). To date, neither echinenone nor canthaxanthin have been isolated from cnidarians or zooxanthellae (Goodwin 1984). The carotenoid composition of *Ar. salina* nauplii and *A. pallida* do not overlap to any



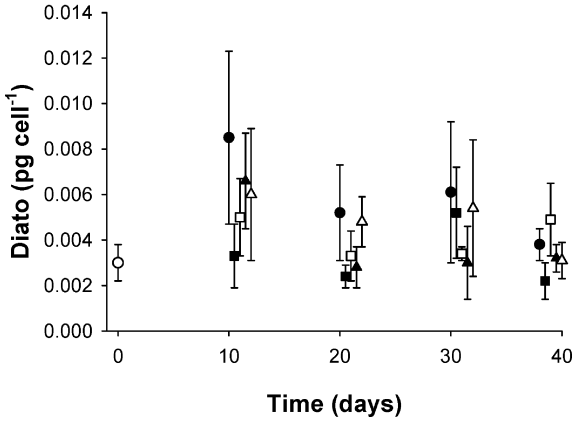
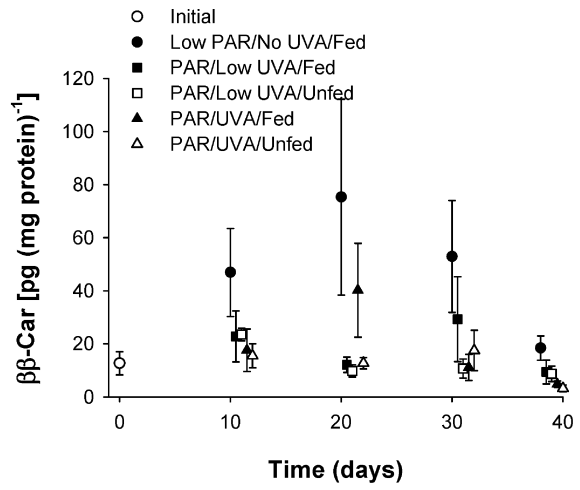


Fig. 4 Mean concentrations of diatoxanthin per milligram of protein of anemone tissue [pg diato (mg protein)$^{-1} \pm SE$] and per zooxanthella (pg of diato cell$^{-1} \pm SE$) over 38 days in *A. pallida* receiving different levels of PAR, UVA and food. For [pg diato (mg protein)$^{-1} \pm SE$], $n = 12$ for initial samples and $n = 6$ in all treatments except $n = 5$ in the following treatments: low PAR/no UVA, fed for 38 days; PAR/low UVA, fed for 10, 30 and 38 days; PAR/low UVA, unfed for 20, 30 and 38 days; PAR/UVA, fed for 30 and 38 days. For (pg of diato cell$^{-1} \pm SE$), $n = 12$ for initial samples and $n = 6$ in all treatments except $n = 5$ in the following treatments: low PAR/no UVA, fed for 30 and 38 days; PAR/low UVA, fed for 10 30 and 38 days; PAR/low UVA, unfed for 20, 30 and 38 days; PAR/UVA, fed for 20, 30 and 38 days; PAR/UVA, unfed for 10, 20 and 30 days



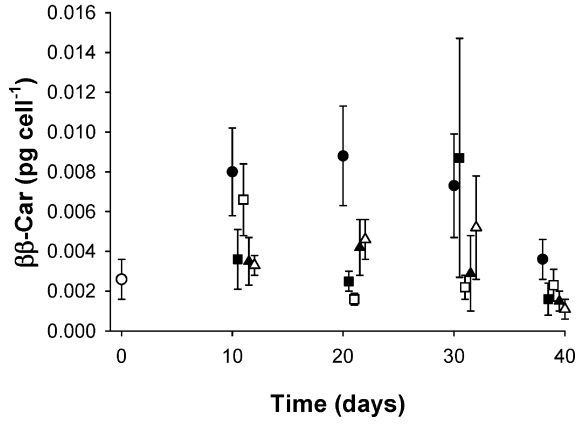


Fig. 5 Mean concentrations of β-carotene per milligram of protein of anemone tissue [pg $\beta\beta$-car (mg protein)$^{-1} \pm SE$] and per zooxanthella (pg of $\beta\beta$-car cell$^{-1} \pm SE$) over 38 days in *A. pallida* receiving different levels of PAR, UVA and food. For [pg $\beta\beta$-car (mg protein)$^{-1} \pm SE$], $n = 12$ for initial samples and $n = 6$ in all treatments except $n = 5$ in the following treatments: low PAR/no UVA, fed for 38 days; PAR/low UVA, fed for 10, 30 and 38 days; PAR/low UVA, unfed for 20, 30 and 38 days; PAR/UVA, fed for 30 and 38 days. For (pg of $\beta\beta$-car cell$^{-1} \pm SE$), $n = 12$ for initial samples and $n = 6$ in all treatments except $n = 5$ in the following treatments: low PAR/no UVA, fed for 30 and 38 days; PAR/low UVA, fed for 10, 30 and 38 days; PAR/low UVA, unfed for 20, 30 and 38 days; PAR/UVA, fed for 20, 30 and 38 days; PAR/UVA, unfed for 10, 20 and 30 days

appreciable extent and thus provide little support for the sequestration of dietary carotenoids. In addition, anemones bleached with the cold-shock technique (Muscatine et al. 1991) and exhibiting low symbiont densities [($< 0.7 \times 10^6$ zooxanthellae (mg protein)$^{-1}$] had little carotenoid pigmentation per mg protein (Mobley and Gleason, unpublished data). It is therefore likely that the majority of carotenoids isolated in *A. pallida* originate from the zooxanthellae and are involved in zooxanthellar photosynthesis. However, it is unclear from this study whether carotenoids present in the zooxanthellae are leaked, sequestered or catabolized in animal tissues. Further study is needed to elucidate the fate of carotenoids obtained from heterotrophic feeding in cnidarians and other symbiotic organisms.

Effects of light and feeding on carotenoids

Recent studies among reef corals link carotenoids present in the zooxanthellae with the mediation of the damaging effects of high irradiance by shunting excess energy away from the reactive photocenter of chlorophyll via non-photochemical quenching thus reducing the potential for chronic photoinhibition and coral bleaching (Ambarsari et al. 1997; Brown et al. 1999;

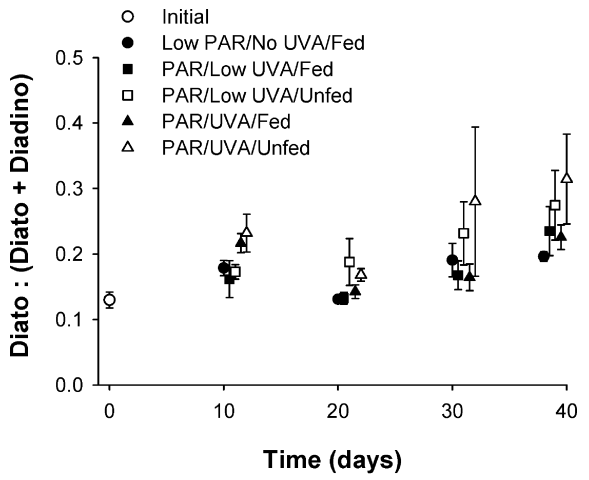


Fig. 6 Mean diatoxanthin production to the total xanthophyll pool [diato: (diato + diadino) ± *SE*] over 38 days in *A. pallida* receiving different levels of PAR and UVA and food. $n = 12$ for initial samples and $n = 6$ in all treatments except $n = 5$ in the following treatments: low PAR/no UVA, fed for 20 and 30 days; PAR/low UVA, fed for 10, 30 and 38 days; PAR/low UVA, unfed for 20, 30 and 38 days; PAR/UVA, fed for 30 and 38 days

Warner et al. 1999; Fitt et al. 2001). Although both high UVR and PAR are implicated in photoinhibition and photo-oxidation of zooxanthellae and the bleaching response of symbiotic organisms (Lesser, 1996; Warner et al. 1996; Hoegh-Guldberg 1999; Hoegh-Guldberg and Jones 1999), changes in spectral composition and intensities of light have not yet been investigated for their effect on the photoprotective properties of carotenoids among symbiotic organisms. UVR is known to induce carotenogenesis in bacteria and fungi and correlates strongly with an increase in antioxidant activity and cell survival (Tada 1993; Sandmann et al. 1998). Götz et al. (1999) also reported net carotenogenesis, chlorophyll synthesis and increased nitrogen uptake in marine phytoplankton as a result of UVA exposure. The combination of UVA and UVB, on the other hand, led to a decrease in the concentration of chlorophyll *a*, diadinoxanthin, fucoxanthin, peridinin and an unknown carotenoid while stimulating xanthophyll cycling and inhibiting nitrogen assimilation (Götz et al. 1999). In other studies of non-symbiotic, photosynthetic dinoflagellates, peridinin and β-carotene provide an additional role as quenchers of singlet oxygen (Pinto et al. 2000).

In this study UVA had little effect on the carotenoid concentrations per cell and per mg protein and likely had little affect on de novo carotenoid synthesis at these experimental light levels. In contrast, a significant increase in xanthophyll cycle activity was observed among higher UVA treatments at 10 days (Fig. 6). This increase is likely related to enhanced photoprotective non-photochemical quenching and antioxidant activity as a result of higher UVA exposure upon initiation of the experiment. Previous studies have shown that diadinoxanthin is rapidly converted into the highly photoprotective diatoxanthin when photosynthetic organisms are exposed to high light intensities (Demers et al. 1991; Young and Frank 1996). The increase in diatoxanthin production strongly correlates with an increase in non-photochemical quenching and photoprotection of zooxanthellae (Ambarsari et al. 1997; Hoegh-Guldberg 1999; Brown et al. 1999). Therefore, xanthophyll cycling provides a rapid response to changes in light intensity without incurring the relatively high costs of carotenogenesis (Demers et al. 1991; Porra et al. 1997).

This initial effect of UVA on xanthophyll activity disappeared by 20 days and may be attributable to upregulation of other photoprotective mechanisms, such as mycosporine-like amino acids (MAAs) and antioxidant enzymes, within zooxanthellae and host animal tissues. Recent experiments by Zudaire and Roy (2001) investigating photoacclimation in the marine diatom, *Thalassiosira weissflogii*, support this hypothesis. Grown under different regimes of UVR for 40 days, *T. weissflogii* show an upregulation of the xanthophyll cycle for the first 16 days of the experiment during which growth rates and photochemical capacity decreased. Following the peak in xanthophyll activity at day 16, xanthophyll cycle activity decreased over the next 13 days and MAA concentrations increased in response to UVR light. These results suggest that MAAs may provide protection against prolonged UVR exposure once the initial lag in net MAA synthesis is overcome. Their build-up over time may account for the reduction in xanthophyll activity observed in our experiment after 10 days in PAR/UVA treatments. Samples were taken for MAA analysis during the experiment and will be analyzed in a companion study. This analysis should reveal whether or not there was net synthesis of MAAs as a response to UVR exposure.

Feeding did not significantly influence carotenoid concentrations per zooxanthellae or per mg protein in experimental anemones. However, a higher ratio of diatoxanthin to total xanthophyll pool was observed at 20, 30 and 38 days in unfed treatments indicating more rapid xanthophyll cycling that may have its origins in nutrient limitation (Fig. 6). When exposed to long-term nitrogen deficiency, plants increase xanthophyll cycling, xanthophylls relative to chlorophyll, non-photochemical quenching and chloroplast-based antioxidant enzymes such as super-oxide dismutase relative to protein content (Logan et al. 1999). These physiological responses indicate that nitrogen deficiency promotes an increase in photo-oxidation and a general decrease in light-harvesting and photosynthetic function in chloroplasts (Logan et al. 1999). Thus, it is likely that host feeding in *A. pallida* influences xanthophyll cycling and prolonged nutrient limitation resulting from inadequate intake of exogenous food sources may increase the susceptibility of zooxanthellae to photo-oxidation and photoinhibition.

In summary, carotenoids in the tissues of *A. pallida* appear to be photosynthetic in origin and supplied by intracellular zooxanthellae. Although many marine organisms may incorporate carotenoids from heterotrophic feeding, this study does not support a dietary

influence on the presence or concentrations of carotenoid pigments within the tissues of *A. pallida*. The diatoxanthin cycle appears to provide primary protection to zooxanthellae against photo-oxidation and photoinhibition from UVA exposure, but the decline in xanthophyll activity after 20 days of this exposure may indicate an increase in additional photoprotective measures such as MAAs in tissues. Finally, similar to what has been observed in terrestrial plants, nutrient deficiency increased photoprotective xanthophyll cycling. This response indicates that symbiotic anthozoans may maintain the ability to rapidly quench oxygen radicals induced by exposure to stresses, such as UVR and high temperature, even when the health of the host is compromised.

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